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Thalictrum minus cell cultures and ABC-like transporter

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

Cultured *Thalictrum minus* cells produce a benzylisoquinoline alkaloid, berberine, in the presence of benzyladenine, and excrete it into the culture medium. *T. minus* cells excluded berberine, even if berberine was exogenously added to the medium, without benzyladenine treatment. Similarly, *T. minus* cells excluded a heterocyclic dye (neutral red) and calcein AM, which is used as a fluorescent probe to detect the drug efflux pump activity by ABC transporters. The addition of several inhibitors of P-glycoprotein, a representative ABC transporter, induced the accumulation in of both berberine and calcein AM ATP-dependent manner. The expression of P-glycoprotein-like ABC transporter genes was also demonstrated. The involvement of ABC transporter in the secretion of berberine in *T. minus* cells is discussed.

Keywords: ABC protein; Berberine; *Thalictrum minus*; *Mdr*; Transport

1. Introduction

Alkaloids comprise one of the largest groups of secondary products, and have divergent chemical structures and diverse biological activities. Plant alkaloids are used in both modern and traditional medicine, e.g., vincristine and taxol are prescribed as anticancer drugs, morphine and scopolamine are used as analgestics, and berberine, a benzylisoquinoline alkaloid, is conventionally used as an antibacterial and antimaleria drug (Yamamoto et al., 1993; Iwasa et al., 1998). These alkaloids also often show strong cytotoxicity to prokaryotic and eukaryotic cells; e.g., vincristine inhibits microtubule formation, and berberine inhibits DNA and protein synthesis (Ghosh et al., 1985). Because of these activities, alkaloids are presumed to play an important role as a biological barrier to protect the plant tissue from pathogens. Indeed, berberine shows strong antimicrobial activity to both Gram-positive and Gram-negative bacteria as well as other microorganisms (Iwasa et al., 1998; Schmeller et al., 1997).

On the other hand, alkaloid-producing plant cells seem to be insensitive to their metabolites. Berberine-producing plant cells must have a mechanism protecting them from the cytotoxicity of berberine, although how it is detoxified in plant cells is still unclear. One of such detoxification mechanisms is probably the sequestration of berberine into vacuoles or the efflux of berberine by the plant cells to keep apart it from the cytosol and also from the nucleus. We have been studying the alkaloid transport mechanism in berberine-producing cultured cells of *Thalictrum minus* and *Coptis japonica* as model systems (Yamamoto et al., 1987; Sato and Yamada, 1984) to elucidate the detoxification mechanism.

We report in this study the high tolerance of cultured *T. minus* cells that produce a large amount of berberine, in comparison with cells that do not have berberine biosynthetic pathway. While cultured *T. minus* cells preferentially excreted the endogenous berberine into the medium, they also excluded berberine exogenously added to the culture, as well as a heterocyclic dye, neutral red, and calcein AM, a fluorescent probe to measure the

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pumping activity of multidrug efflux transporter in an ATP-dependent manner. Further analyses using inhibitors of ATP-binding cassette (ABC) proteins have suggested that a member of ABC transporters is involved in the transport of berberine in the *Thalictrum* cells.

2. Results

The cultured *T. minus* cells established in this study (line Tm103) showed a similar inducibility of berberine by the addition of benzyladenine (BA) as reported by Hara et al. (1993). As shown in Fig. 1, Tm103 cells showed marked production of berberine in the presence of BA and excreted it into medium.

Using this cell line, the effect of berberine on the cell growth was examined in the comparison with *Lithospermum* cells which have no berberine biosynthetic pathway (Figs. 2A–C). Berberine added to the medium strongly inhibited the growth of *L. erythrorhizon*, whereas the cell growth of *T. minus* was not largely affected by exogenous berberine addition regardless of

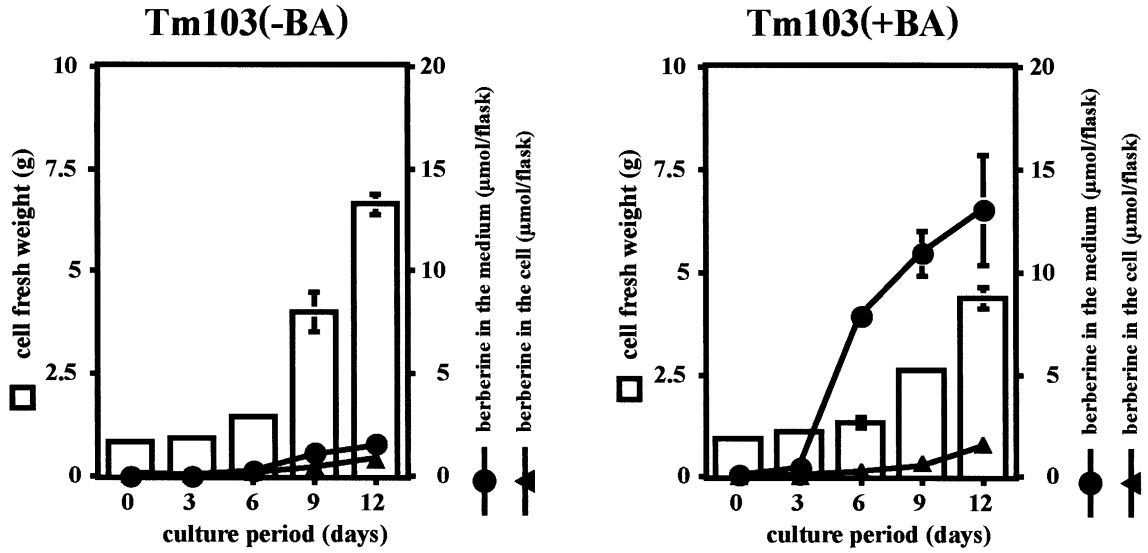

Fig. 1. Cell growth and berberine production in cultured cells of *Thalictrum minus*. Fresh cells (1.0 g) were inoculated in culture medium in the presence or absence of BA. Strain Tm103 was berberine-producing cell line.

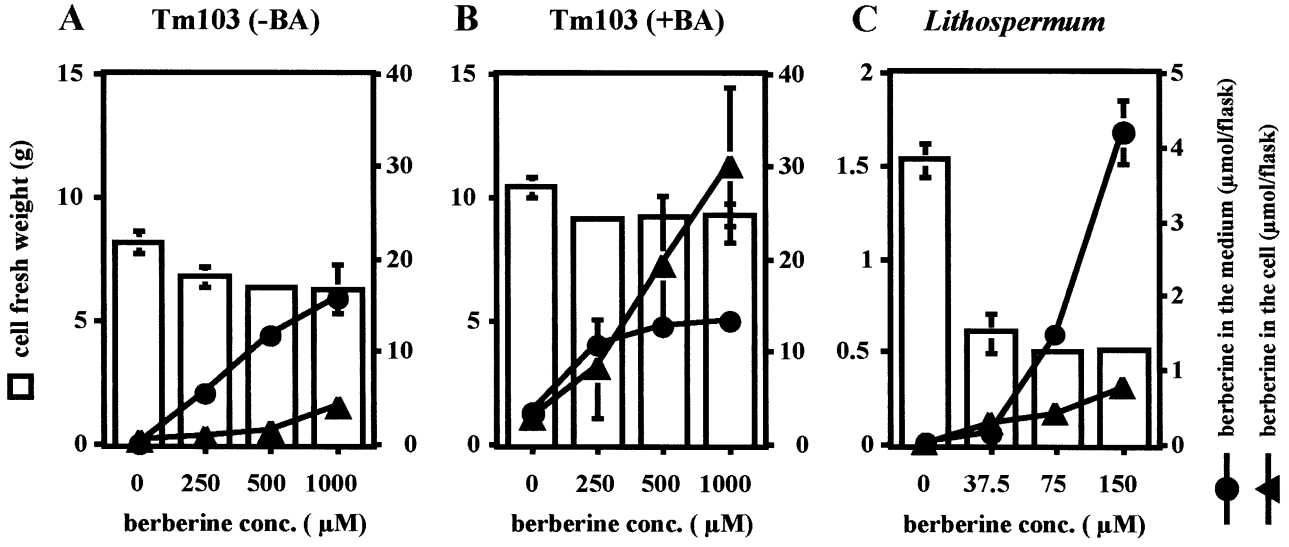

Fig. 2. Effects of berberine on the growth of cultured cells of *Thalictrum minus* and *Lithospermum erythrorhizon*. Fresh cells (1.0 g) were inoculated in culture medium (30 ml) containing berberine and cultured for two weeks on a rotary shaker. Cell fresh weight represents the growth of the plant cells harvested after the cultivation.

the berberine production. This suggested that *T. minus* was potentially tolerant to its native secondary metabolite, berberine, while it would be toxic to plant cells that do not have berberine biosynthetic pathway. Tm103 cells showed low or little accumulation of berberine in the cells, while in the presence of BA showed moderate accumulation of berberine in the cells. These results suggested that *T. minus* cells had the potential to exclude exogenously added berberine in spite of the berberine production ability. The tolerance of *T. minus* to berberine was more than 20-fold higher than that of *Lithospermum* cells.

To examine the exclusion activity of *T. minus* cells, we used neutral red, an aromatic red dye, which was also an amine that accumulates in acidic compartments and conventionally applied to stain plant vacuoles. As Fig. 3 shows, *T. minus* cells excluded neutral red independently from BA, whereas *C. japonica* cells were stained. The pH values of *T. minus* cells, which were measured according to the method of Kurkdjian and Guern (1981), showed normal acidic range 5.5 under the both culture conditions with and without BA, suggesting that vacuolar pH was not the cause of non-staining of *T. minus* cells. Further experiment to evaluate the exclusion activity of plasma membrane using protoplasts and vacuoles, indicated that plasma membrane of *T. minus* cells was responsible for the efflux of neutral red and isolated vacuoles were clearly stained by neutral red as other plant vacuoles.

Excretion of chemicals have been well studied in cancer cells, in which ABC transporters localize at plasma membrane and decrease the intracellular concentrations of drugs. In fact, neutral red and berberine exhibit a typical structural feature for the preferable substrate of MDR (multidrug resistance protein)-type ABC transporter, such as P-glycoprotein, a gene product of *mdr1* (multidrug resistance protein 1), and berberine was recognized as a substrate of human P-glycoprotein (our unpublished data). Thus, we examined whether or not *T. minus* cells have the drug efflux activity at the plasma membrane using a fluorescent probe, calcein AM, which is conventionally used to measure the efflux activity of ABC transporters (Tiberghien and Loor, 1996).

As Fig. 4A shows, fluorescent microscopy revealed different staining pattern between *T. minus* cells and tobacco that was used as a control. Whereas tobacco cells showed strong fluorescence, *T. minus* cells had almost no fluorescence. Since medium of *T. minus* cells showed fluorescence, calcein AM was once hydrolyzed by intracellular esterases and secreted calcein having strong fluorescence into the medium. This result suggested that an ABC-type efflux-transporter is functioning in *T. minus* cells, while not in tobacco cells. Accumulation of calcein by addition of several inhibitors, i. e. nifedipine, verapamil and vanadate supported this idea (Fig. 4B). Vanadate is a membrane ATPase inhibitor (Ambudkar et al., 1992), and nifedipine and verapamil are established inhibitors of P-glycoprotein (Wigler, 1996).

Since the presence of ABC-type transporter at plasma membrane of *T. minus* cells was suggested, we examined the effect of those inhibitors of P-glycoprotein on the berberine efflux in *T. minus* cells line Tm103. While

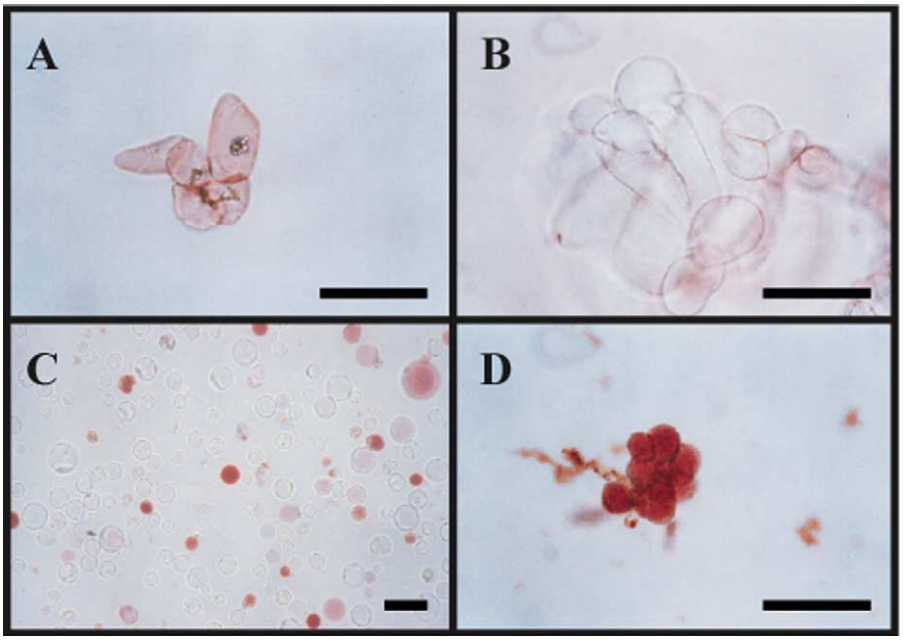

Fig. 3. Neutral red staining of *Thalictrum minus* cells. A, Tm103 (-BA); B, Tm103 (+BA); C, protoplasts and vacuoles (*T. minus* line Tm103); D, *Coptis japonica*.

Tm103 cells did not accumulate berberine added to the culture medium in the control, several inhibitors of ABC proteins, i. e., verapamil, cyclosporin A, and quinidine, induced the accumulation of calcein as shown in Fig. 5. These data suggested that an ABC transporter was also involved in the efflux of exogenously added berberine from the cells, and would be responsible for the secretion of the endogenous berberine.

To examine the involvement of MDR-type ABC transporter, we analyzed the expression pattern of MDR-type genes, which form an important drug efflux pump subfamily in ABC transporters (Martinoia et al., 2002). First, we amplified cDNA fragments corresponding to the nucleotide binding fold of MDR-type ABC transporters. In total, five fragments of 320 bp were isolated, which show striking amino acid identity each other (Fig. 6). We selected two major molecular species (Tm-t10 and Tm-t15) and observed the expression pattern during the culture (Fig. 7). The expression levels of three other molecular species appeared to be much lower than Tm-t10 and Tm-t15 (data not shown). *T. minus* cells expressed both molecular species regardless of BA-induced berberine production. This constant expression of Tm-t10 and Tm-t15 was consistent with the berberine efflux activity of the cells. The size (ca. 5 kb) is slightly larger than the conventional MDR-like

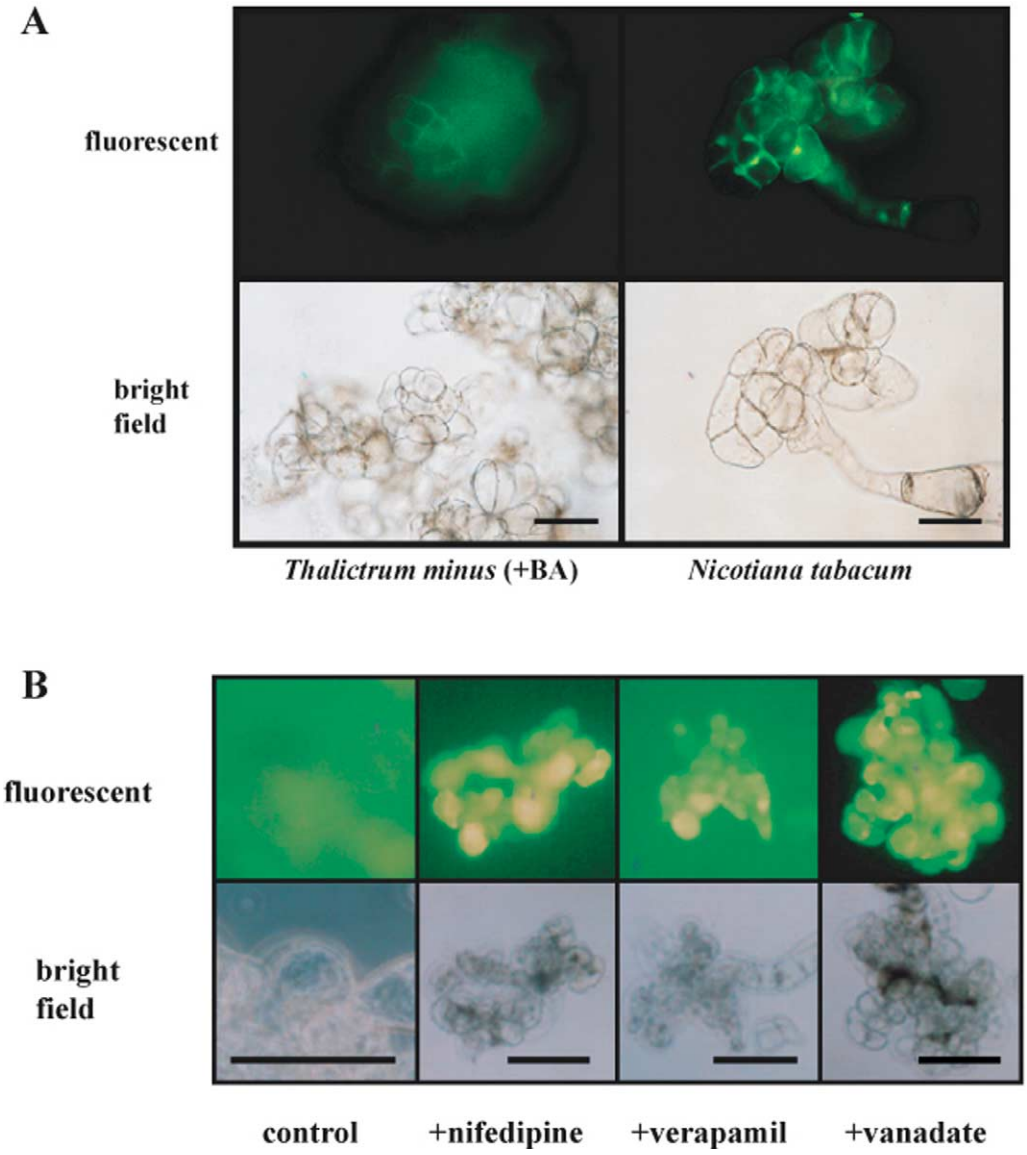

Fig. 4. Fluorescent micrographs of cultured *Thalictrum minus* (+BA) and *Nicotiana tabacum* cells incubated with calcein AM. Fresh cells were incubated in the conditioned medium with calcein AM (final concentration: 2.5 μM), for 30 minutes at 25 °C (A). Each inhibitor was added 30 min before the addition of calcein AM (B). Nifedipine, 50 μM; verapamil, 50 μM; vanadate, 1 mM. Bars, 100 μm.

gene transcript. The induction of berberine production is regulated at the reaction steps of norcoclaurine 6-*O*-methyltransferase (Hara et al., 1995a) and tetrahydroberberine oxidase (Hara et al., 1995b), whose genes are not yet isolated from *T. minus*, although the inducibility of berberine production does not have a direct correlation with the berberine efflux (Fig. 2).

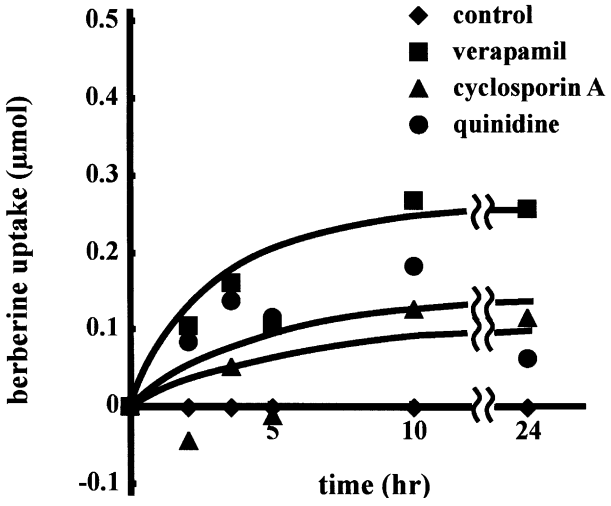

Fig. 5. Effects of various inhibitors on berberine efflux by *Thalictrum minus* (-BA) cells. Each inhibitor was added 30 min before the addition of berberine. Fresh cells (0.5 g) were incubated in conditioned medium (10 ml) with berberine (final concentration: 200 μM) and inhibitors at 25 °C, and the berberine uptake was monitored in time course. Verapamil 50 μM; cyclosporine A, 10 μM; quinidine, 50 μM.

3. Discussion

In this study, we demonstrated that berberine had strong cytotoxicity to plant cells that do not have berberine biosynthetic pathway, while berberine-producing plant cells, *T. minus* had a clear tolerance to it regardless of berberine production (Fig. 2). This suggests that there is a species-specific detoxification mechanism for plant cells. Whereas other berberine producing *Coptis japonica* cells have berberine accumulation inside cells, especially vacuoles (Sato et al., 1990; Sato and Yamada, 1984; Sakai et al., 2002), *T. minus* cells seems to excrete alkaloid from the cells to prevent the accumulation in cytosol.

The transport of secondary metabolites in plant cells are often reported to be energy-dependent (Yamamoto et al., 1987; Deus-Neumann and Zenk 1984, 1986). Actually, the efflux activity of endogenous berberine by *T. minus* cells was very sensitive to the membrane ATPase inhibitor, vanadate (data not shown). Furthermore, various inhibitors of P-glycoprotein decreased the exogenous berberine transport activity of the cells (Fig. 5). P-glycoprotein, discovered in human cancer cells, was a large membrane protein responsible for multiple drug resistance against anti-cancer drugs by effluxing these chemicals of divergent structures (Ueda et al., 1987). Inhibition of excretion of exogenously added berberine in *T. minus* cells and transport of berberine by human P-glycoprotein suggested that a similar mechanism of human P-glycoprotein may be involved in berberine efflux by *T. minus* cells. Interestingly, there

Fig. 6. Alignment of putative amino acid sequences of cDNA fragments amplified by RT-PCR from *Thalictrum minus* cells (line Tm103). Tm-t6, 10, 15, 17 and 24 are the fragments isolated from *T. minus* in this study. AtPGP1-1and AtPGP1-2 are the nucleotide-binding fold of *Arabidopsis thaliana* P-glycoprotein like gene, AtPGP1 (Dudler and Hertig, 1992).

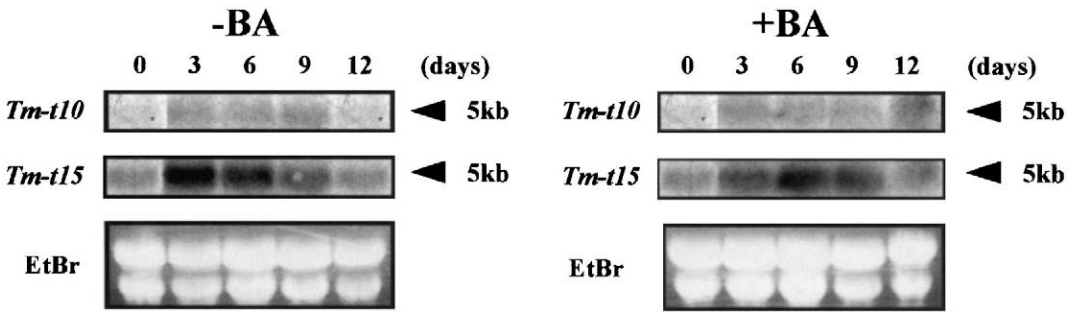

Fig. 7. RNA gel blot analyses of cultured cells of *Thalictrum minus* (line Tm103). *Tm-mdr* fragments (Tm-t10 and t15, each 320 bp) were used as hybridization probes. Each lane contains 15 μg of total RNA fraction. The bottom panels show the ribosomal RNA detected with ethidium bromide (load control).

was a clear difference between *T. minus* cells and berberine-non-producing cells of tobacco, for the secretion of calcein AM used as a fluorescent probe of ABC transporter. This result suggested that the presence of ABC transporters responsible for effluxing these compounds was unique to *T. minus* cells. The existence and expression of such ABC transporters in *T. minus* were also demonstrated in this study (Figs. 6 and 7). Our data suggested that ABC transporters were localized at the plasma membrane of *T. minus* cells and involved in efflux of aromatic compounds, although further studies are needed to prove that such ABC transporters are directly exporting the endogenous alkaloid, berberine, in this plant cells.

Recently, we have isolated a cDNA of ABC protein (Cjmdr1) encoding 1289 amino acids, which also belongs to the MDR subfamily, from cultured *C. japonica* cells (Yazaki et al., 2001). Functional analysis of this ABC transporter indicated that it shows the influx activity of berberine (Shitan et al., unpublished data). Thus, the information of ABC transporter of *T. minus* would be useful to understand the efflux mechanism of berberine in *T. minus* cells. Plant ABC protein is a newly developing research area (Rea, 1999; Theodoulou, 2000; Davies and Coleman, 2000; Sánchez-Fernández et al., 2001; Martinoia et al., 2002; Sidler et al., 1998). Molecular characterization of berberine transport in *T. minus* cells should provide us further information about the ABC proteins involved in alkaloid transport in plant cells.

4. Experimental

4.1. Cell suspension culture

The berberine-producing and berberine-non-producing cultured *Thalictrum* minus cells, which were originally induced from petioles of *T. minus* L. var. *hypoleucum* Miq., were maintained as described elsewhere (Nakagawa et al., 1984). Berberine was added upon inoculation of the cells and harvested two weeks after inoculation. The cultured cells of *Lithospermum erythrorhizon* (line M18) and *Nicotiana tabacum* (cv. SamsunNN, line NII) were also continuously subcultured according to the methods by Yazaki et al. (1997) and Takeda et al. (1990), respectively. Protoplasts and vacuoles from *T. minus* cells prepared according to the method reported previously (Yazaki et al., 1995).

4.2. Toxicity of berberine to various plant cells

To evaluate the sensitivity of various plant cells to berberine, 1.0 g fresh weight of cells were inoculated in the medium containing various concentrations of berberine (30 ml) and cultured on a rotary shaker (100 rpm) for 14 days in darkness at 25 °C. Cells were harvested by filtration through Miracloth (CALBIOCHEM. Co.) and the fresh weight was measured. Berberine concentration in the medium was determined by the measurement of the absorbance of the supernatant at 420 nm. Berberine concentration in the cell was determined by HPLC after it was extracted into methanol containing HCl (Hara et al., 1993). Berberine was detected by reverse phase HPLC (mobile phase, H_2O (50 mM L(+)-tartaric acid, 10 mM SDS): acetonitrile: methanol = 100: 130: 33; column, ODS-80$_{TM}$ (4.6×250 mm; TSK-GEL); flow rate, 1.2 ml/min; detection, absorbance measurement at 260 nm). Experiments were done in triplicate.

4.3. Inhibitor experiments

Prior to the addition of berberine, *T. minus* cells (0.5 g) were treated with inhibitors (vanadate, 1 mM; verapamil, 50 μM; nifedipine, 50 μM; cyclosporin A, 10 μM; quinidine, 50 μM) in 10 ml of conditioned medium for 30 min. After addition of calcein AM (final concentration: 2.5 μM) or berberine (final concentration: 200 μM), cells were incubated at 100 rpm at 25 °C and the uptake was measured. Sodium vanadate was depolymerized before use according to the method of Goodno (1979). Quinidine, nifedipine, and cyclosporin A were dissolved in 50 μl DMSO solution, and added to the medium. In the control, 50 μl of DMSO or H_2O was added, to confirm no effect on DMSO did not affect the calcein AM and berberine uptake and on cell viability at this concentration. Fluorescence of calcein was observed with Axioskop 2 (Zeiss) with an excitation filter of 470 nm and a barrier filter of 500–530 nm (band path).

4.4. Isolation of Tm-mdr fragments

Total RNA prepared from cultured *T. minus* cells was reverse-transcribed with M-MLV reverse transcriptase (NEB). Nested PCR was carried out with Taq DNA polymerase (TaKaRa), with the DNA-RNA hybrid template, and two sets of degenerate primers designed from the highly conserved amino acid sequence in the nucleotide-binding fold of ABC proteins (Yazaki et al., 2001).

4.5. RNA gel blot analysis

Total RNA of *T. minus* was prepared from 200 mg cultured cells by RNeasy Plant Mini Kit (QIAGEN). Agarose gel electrophoresis, RNA transfer onto Hybond-N+ membranes (Amersham Pharmacia), and hybridization with the 0.3 kb *Tm-mdr* fragments were carried out with standard procedure. The last wash was performed with 0.2×SSC/0.1% SDS at 60 °C.

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